AIRBORNE MYCOTOXIN SAMPLING AND SCREENING ANALYSIS

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ABSTRACT
The indoor mycotoxins inhalation exposure of patients (n=25) was studied using a high-volume air sampler (60 cfm x 24 h) with a micropore-paper filter (8x11 inches). The filters were evaluated for cytotoxicity caused by mycotoxins using the MTT-cell culture bioassay and by culture identification. A subset of samples was analyzed with an enzyme-immuno assay for occurrence of macrocyclic trichothecenes produced by *Stachybotrys chartarum* and HPLC-DAD and GC-MS analyses for different mycotoxins. Highly toxic air samples ($IC_{50} \leq$ 31 mg/ml) were found in seven cases; moderate toxicities (IC_{50} > 31 to ≤ 125 mg/ml) in 14 cases, and four cases were not toxic compared to controls. The subset testing demonstrated that macrocyclic trichothecenes and other mycotoxins could become airborne. In conclusion, an inhalation risk could be confirmed (84% of cases) with the 24-hour high volume air sampling test method due to the detection of airborne cytotoxic fungal particles and specific mycotoxins, including trichothecenes produced by *Stachybotrys* fungi.

INDEX TERMS
Indoor mycotoxins, Cytotoxicity, MTT bioassay, *Stachybotrys c.*, Inhalation risk

INTRODUCTION
Mycotoxins consist of a group of more than 400 chemical compounds produced by several fungal genera and species. The production of mycotoxins is dependent on the toxigenicity of the particular fungal strain, the composition of the substrate and various external factors such as water activity, pH-value, temperature, oxygen and the presence of competitive micro-organisms. In indoor environments the occurrence of several fungal species and mycotoxins has been demonstrated, typically after significant water damage and with high moisture on cellulose materials. Mycotoxins produced by fungi (i.e., *Stachybotrys chartarum* (S.c.)) may be causal agents in non-agricultural exposures that are associated with immune suppression and central nervous system disorders. Toxic effects of mycotoxins in humans have been discussed primarily in the context of ingestion related poisoning, but to a much lesser degree related to inhalation risk. This appears to be due to prior sampling difficulties and analytical limitations. In the past indoor air microbial investigations and exposure assessments have generally relied on mycological identification using viable- and non-viable methods (culture ID, fungal spore counts, bulk and air sampling), which may give qualitative or quantitative information about the presence of particular fungi, but not its particular properties (toxicity?). Laboratory chemical analyses of bulk materials from contamited homes do not necessarily reflect the true conditions in the buildings („in vivo toxicity“) and may therefore be misleading. Clinical experience shows that a variety of patients working or living in buildings

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with certain type of fungal contamination present with symptoms and signs that are uniquely different from the simple allergy model, but rather suggest a toxicological pathology based on laboratory analysis and neuro-behavioral evaluations among other tests. This prompted us to study their inhalation exposure. The cases that were investigated were selected based on a specialty clinical assessment of symptoms and abnormalities described in detail elsewhere (Johanning et al. 1999) and the presence of widespread fungal contamination, including *Stachybotrys, Penicillium, Aspergillus, Trichoderma, Chaetomium, and Paecilomyces*. Typical complaints and findings of such case patients were significant upper and/or lower airway symptoms, skin rashes, headaches, excessive fatigue, cognitive problems (memory, concentration, and irritability, etc.), laboratory findings showed lower counts of mature lymphocytes (CD3) and of some lymphocyte subsets, or immune globulin elevations (IGG and IGA to specific fungi, including Stachybotrys c.) and significant abnormalities in formal standardized neurocognitive testing.

MATERIAL AND METHODS

Air sampling

Indoor airborne particles in homes or offices (n=25) of patients were collected using a high-volume air sampler (sampling rate 60 cfm) with a filter paper (8x10 inches; 20 µm pore-size) (n=56) that run for 24 h in various rooms with visible and confirmed fungal contamination. Controls consisted of blank filter paper send for analysis. The air filters were dry and with no visible fungal growth. Fungal spore counts (Burkard, 3 min 10l) were taken before and after high volume sampling.

Cell culture

Swine kidney monolayer cells (SK), known for high sensitive towards mycotoxins, were used as target cells (Hanelt, Gareis, & Kollarczik 1994). The cells were maintained in 150 cm^2 tissue culture flasks (TPP, Renner GmbH Darmstadt) in 100 ml medium (MEM, Minimum Essential Medium with Earle's Salts, Sigma-Aldrich) supplemented with 200 IU Penicillin/ml and 200 µg streptomycin/ml at 37 °C in humidified atmosphere with 5 %CO_2. For the assay, the cells were harvested with trypsin/EDTA (1:10,v:v) in phosphate buffered saline (PBS) and plated in flat bottom 96-microtiter plates (TPP, Renner GmbH Darmstadt) in 100 µl culture medium (MEM, Minimum Essential Medium with Earle's Salts supplemented with 200 IU Penicillin/ml, 200 µg streptomycin/ml, 1,7 % ethanol, 0,3 % DMSO and 10% FCS) at densities of about 5 x 10^5 cells/ml. 100 µl of the culture medium (control) or culture medium containing the sample extracts prepared as described below were added to the wells with duplicates for each concentration at the same time. This way each well contained 200 µl of medium with a final concentration of 5 % FCS. Eight wells of the plate remained without cells and served as blanks. The plates were then incubated for 48 hours at 37 °C in a humidified atmosphere with 5% CO_2.

MTT-Bioassay

The colorimetric tetrazolium MTT cleavage test has been described as a sensitive bioassay for the evaluation of a series of mycotoxins and has been used as diagnostic tool in the screening of a variety of mycotoxin-contaminated materials such as food and feed, fungal cultures and samples from indoor environments (Hanelt, Gareis, & Kollarczik 1994); (Gareis 1994); (Johanning, Gareis, & Dietrich 2000). The principle of this bioassay is based on the transformation of the yellow tetrazolium salt MTT by viable, living cells (via mitochondrial dehydrogenase) to purple formazans (Mossmann 1983). The assay carried out in the study presented here was performed as previously described by Gareis (Gareis, Johanning, & Dietrich 1999). Briefly, at the end of the incubation period a volume of 20 µl of the MTT

stock solution (3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; Sigma-Aldrich) in PBS at a final concentration of 5 mg/ml was added to each well and the plates incubated for another 4 hours at 37 °C in 5% CO_2. Supernatant was then removed using a multi-channel micro pipette and 100 µl DMSO was added to each well in order to dissolve the dark formazan crystals. The optical density of each well was measured spectrometrically with an ELISA-Reader (anthos 2010) at a wavelength of 510 nm and data calculated with MicroWin 2000 (Mikrotek Laboratories, Overath). Mean extinction values and standard deviations of each sample concentration were compared with those of the corresponding control and expressed as % cleavage activity in comparison to cell controls (100 %). The cytotoxic endpoints, i.e. the minimum concentration of the test reagents measured to cause toxic effects were determined on the basis of the IC_{50} value (Inhibitory Concentration $_{50}$= concentration resulting in 50% inhibition of the MTT cleavage activity).

Paper filters from air sampling

Two g of the air filter paper material were crushed by cutting and soaked in 40 ml of methanol overnight. The paper was then filtrated and extracted twice with 40 ml chloroform and 40 ml methanol for 30 minutes each. The combined filtrates were dried under rotary evaporation and the remaining residue transferred with 2 x 3ml acetone/methanol (2:1,v:v) to small test tubes. The sample was concentrated under a gentle stream of nitrogen and dissolved in culture medium without FCS (MEM supplemented with 200 IU Penicillin/ml, 200 µg streptomycin/ml, 1,7 % ethanol, 0,3 % DMSO). Serial log 2 dilutions using the cell culture medium were prepared and transferred to the cell culture plates. Final concentrations of the crude extract of the paper filters ranged from 3,9 to 1000 mg/ml of cell culture medium. For recovery experiments, non-exposed paper filters were spiked with 1 µg each of satratoxin G and H dissolved in ethanol. After drying and evaporation of the solvent, papers were extracted and concentrations ranging from 0.5 to 0.0019 µg/ml tested in the MTT assay as described above.

Chemical analysis

Small air filter pieces of approximately 10 cm^2 were extracted cleaned with PEI-silica and were analyzed with HPLC-DAD. Hydrolyzed extracts were analyzed with GC with simultaneous MS and MS/MS detection (Nielsen & Thrane, 2001). No fungal metabolites were detected from the reference filters.

RESULTS

Recovery of satratoxin from filter papers

The lowest concentration (IC_{50}) of satratoxin standard mycotoxins to cause cytotoxic effects in the MTT-bioassay was found to be 0,008 µg/ml (satratoxin G) and 0.02 µg/ml (satratoxin H). Paper filters spiked with the toxins reacted positively at concentrations as low as 15 mg/ml and 31 mg/ml which is equivalent to recovery rates of 97 to 85 % for the both mycotoxins (**table 1**).

Table 1. Cytotoxicity of satratoxin and recovery rates from paper filters

	IC_{50}* µg mycotoxin/ml	IC_{50}* mg sample aliquot/ml	Recovery Rate
Filter paper control		250	
Filter paper + Satratoxin G	0,0079	15,62	97 %
Filter paper + Satratoxin H	0,0156	31,25	85%
Satratoxin G Standard	0,0081		
Satratoxin H Standard	0,0183		

IC 50 (Inhibitory concentration 50 %): lowest concentration measured to reduce the MTT-cleavage activity to 50 %

Cytotoxicity of air filter

The IC_{50} values of the matrix, i.e. crude extracts from non-exposed filter controls proved to be 250 to 500 mg of sample aliquot per ml of cell culture medium. In 59 field samples originating from 25 case investigations IC_{50} values range between 2 and 250 mg/ml, thus demonstrating varying degrees of cytotoxicity (Table 2).

Table 2. Results of cytotoxicity testing of paper filters from 25 case investigations

No	Loc.	IC_{50} mg/ml	Fungi	Mycotoxins
1	Tx A	2	**Stach +++,** Clad +++, Muc +++, Pen +, Tri +	HPLC-DAD:- Spiriocyclic dimanes-metabolites with UV-spectra id.to MER-NF 5003's; GC/MS:Verrucarol
	B	4	**Stach +++,** Clad +++, Tri ++, Pen ++, Asp ++, Muc +	HPLC-DAD:- Spiriocyclic dimanes- metabolites with UV-spectra id.to MER-NF 5003's; GC/MS:Verrucarol
2	NY	15	Asp +++, Cand +,Clad +,Geo ++,Mon +, Pen+	
3	Ms A	31	1 Geo, 1 Hef	
	B	31	1 Asp, 1 Pen	
4	NJ	31	Tri+++,Clad+++,**Stach +++,**Pen+++, Trich+++	
5	Ms	15	Asp +, Acr +, Paec +, Yeast ++	
	B	62	Pen ++, Asp +	
6	NY A	31	Negative	
	B	62	1 Asp	
7	Pa A	31	Asp ++, **Stach +**, Pen +, Fus +, Epi +, Alt +, Clad (+), Paec ++, Hef ++	HPLC-DAD:No fungal metabolites - Antracene like spectra
	B	62	Asp +, Paec +, Clad +, Hef +	HPLC-DAD:No fungal metabolites - Antracene like spectra
8	Al A	62	Alt +++, Clad +++, Epi ++, Pen ++, Rhiz +, Muc (+), Chae +	
	B	62	Tri ++, Alt ++, Rhiz +, Clad ++, Pen +, Asp +, Epi +, Chae +	
9	Ks A	62	Epi ++, Alt ++, Fus +, Clad+++,Bot+,Pen +++, Asp +++, Paec ++, Sync +,Chae +,Tri +, Nig+	
	B	62	Tri +, Clad +++, Asp ++, **Stach +,** Chae ++, Pen +, Alt +, Paec +, Acr +	
10	NJ A	62	Clad ++, Epi +, Pen ++, Asp ++, Acr +	
	B	62	Clad (+), Pen +, **Stach +,** Asp +, Paec +	
	C	125	Clad +, Pen +++, Chae +, Asp +, Tri +	
11	Mi A	62	Pen +, Clad +++, Asp +	
	B	125	Pen ++, Asp ++, Paec +, Eur +	
12	NY A	62	Pen +++, Asp ++, Clad +, Tri (+)	
	B	62	Pen +++, Asp ++, Clad (+)	
	C	125	Asp +, Fus (+)	
	D	125	Pen +, Asp +, Paec +, Geo (+)	
1	Ca A	62	Pen ++, Asp +, Clad ++, Alt +, Acr +, Eur +	

3				
	B	125	3 K Asp, 1 K Pen	
	C	125	1 K Clad	
14	Az A	125	Negative	
	B	125	Negative	
	C	125	Tri ++, Pen +, Asp +, **Stach +**	
	D	125	Tri ++, Asp +++, Pen +	
	E	125	Negative	
	F	125	1 Asp	
15	Ca A	125	Clad +, Pen +++	
	B	125	Alt +, Asp +, Clad +++, Paec +, Pen +++	
16	NC A	125	Negative	
	B	125	Negative	
	C	125	Clad ++, Pen ++, Asp ++,Alt ++, Paec +, Tri +	
17	Ct A	125	Tri ++, Rhiz +, Pen +++, Yeast +, Paec +, Moni +, Fus +	
	B	125	Tri +, Pen +++, Asp +, Mon +	
18	Al A	125	Asp +, Chae (+), Clad ++, Fus +, Pen +++, **Stach +**	
	B	125	Asp ++, Clad +, Fus (+), Pen +++	
19	Mass A	125	Asp +++, Pen +, Clad +	
	B	125	Asp +++, Pen +	
	C	250	Asp +++, Clad +, Pen ++, Bot +	
20	Oh A	125	Pen +++, Chae +, Asp ++, Clad +, Moni (+)	HPLC-DAD:- trace of spiriocycles; GC/MS:- Not detected
	B	250	Pen ++, Asp ++, **Stach +,** Clad +, Mon +	HPLC-DAD:- trace of spirocycles; GC/MS:- trace of trichodermol
21	Ca A	62	Clad +, Pen +, Asp ++, Cand +, Ver (+)	
	B	125	Clad +++, Muc +, Alt +, Asp +, Sac +, Pen +, Ver (+), Fus (+)	
	C	125	Clad ++, Asp +, Pen +, Ver (+)	
	D	500	Clad +++, Pen +, Asp (+), Fus (+)	
22	Oh A	250	Pen ++, Asp ++, Tri +, Geo +, Paec +	
	B	250	Asp (+), Pen (+), Paec (+), Tri (+),Moni (+)	
23	NY	250	Pen +++, Asp (+), Tri +, Muc +	
24	Tx A	250	Chae +, Tri +	HPLC/DAD:-interesting peaks ?; GC-MS:- Not detected; Ro-A-equivalents:< 1 ng/g
	B	250	1 Paec	HPLC/DAD:-interesting peaks?; GC/MS:- not detected;Ro-A equivalents:< 1 ng/g
25	Pa A	250	Pen +++ ,Asp +++, Alt +, Clad +, **Stach++**	
	B	250	Asp +++, Pen +++, Clad +, **Stach ++,** Paec +, Muc +	

Air samples with high airborne levels of toxicity ($IC_{50} \leq 31$ mg/ml) were detected in seven cases of the building investigations. Moderate levels of toxicity ($IC_{50} > 31$ to ≤ 125 mg/ml) were noted in filter papers from 14 cases, while the samples from the remaining four cases appeared non-toxic compared to the controls. *A Stachybotrys chartarum* was identified in the filter papers from ten cases. The presence of *S. chartarum* detected by culture methods was however not always associated with detectable cytotoxic mycotoxins. In the extremely toxic samples from case 1 (Tx) the occurrence of macrocyclic trichothecenes could be determined by GC-MS analysis. These results clearly demonstrate that macrocyclic trichothecenes could become airborne and an exposure risk could not be ruled out. However, no mycotoxins were detected in the toxic filter paper from case 7 (Pa), which was contaminated with different

fungal spores including *S. chartarum.* The origin of the toxic properties is not known, but it appears that the toxicity measured was due to toxic compounds other than macrocyclic trichothecenes or mycotoxins that can be detected by HPLC-DAD. *S. chartarum* was also found in filter samples which proved to be not cytotoxic (case No. 25 PA2). This indicates the presence of non-toxigenic S.c. strains or the non-production of cytotoxic mycotoxins by toxigenic strains in the given
environment.

CONCLUSION

In conclusion, airborne mycotoxin exposure and an inhalation risk could be confirmed in 84% of all investigated cases with the 24-hour high volume air sampling test method based on the detection of cytotoxic fungal particles utilizing the MTT-bioassay and the finding of specific mycotoxins, including trichothecenes by confirmation chemical analysis. The key knowledge obtained in the present study is that air filters from problem environments showed a variable range of toxicity levels. The MTT-assay with the SK target cell line was shown to be useful for the toxicity screening of true field samples. The cells are highly sensitive to mycotoxins and could be challenged with various amounts of crude extracts from filter papers up to 250 mg sample aliquot/ml. Thus a distinction between toxic air filters and non-toxic air filters is possible when comparing exposed filters with controls. From earlier investigations it was known that the level of toxicity obtained by the MTT-bioassay in samples contaminated with or without *Stachybotrys chartarum* was found to be highly correlated with the amount of macrocyclic trichothecenes detected by use of the EIA and/or physical-chemical methods (Gareis, Johanning, & Dietrich 1999). Furthermore, observed toxicities in samples not being moulded with *Stachybotrys chartarum* indicated the presence of cytotoxic mycotoxins produced by other molds and/or other toxic compounds. These results suggest the usefulness of the MTT cytotoxicity assay as an additional and important diagnostic tool for mycotoxins exposure assessment in problem buildings. The highly toxic air filter results from our case investigations clearly support the hypothesis of airborne mycotoxin exposure and related inhalation risks to patients in indoor environments with known toxigenic fungi.

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